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AGGREGATION OF HORSESHOE CRAB (*LIMULUS POLYPHEMUS*) AMEBOCYTES AND REVERSIBLE INHIBITION OF AGGREGATION BY EDTA[1]

DIANNE M. KENNEY, FRANK A. BELAMARICH AND DAVID SHEPRO

Boston University Graduate School, Boston, Massachusetts and Marine Biological Laboratory, Woods Hole, Massachusetts

Amebocytes comprise over 99 per cent of the cells in the hemolymph (blood) of the horseshoe crab, *Limulus polyphemus* (Fahrenbach, 1970). In its circulating phase, the amebocyte is a large, oval shaped, nucleated cell with characteristic cytoplasmic granules that obscure the nucleus. When removed from the circulation amebocytes rapidly aggregate and undergo vast morphological alterations including swelling, loss of granules, vacuolization, formation of filamentous pseudopods, and contraction (Levin and Bang, 1964b; Dumont, Anderson and Winner, 1966).

Functional parallels can be drawn between *Limulus* amebocytes (Levin and Bang, 1966) and mammalian platelets, and non-mammalian thrombocytes (Belamarich, Shepro, Fusari and Kien, 1966), all hemostatic cells specialized to aggregate in response to injury (Loeb, 1927). Unlike vertebrate coagulation that involves cell aggregation in addition to the formation of fibrin, the stemming of blood flow from injured *Limulus* vessels is solely due to the aggregation and contraction of amebocytes (Howell, 1885; Loeb, 1903–04, 1927; Maluf, 1939).

The biochemical aspects of amebocyte aggregation are not well understood. An endotoxin clottable protein originating from amebocytes was identified and its properties are being investigated (Levin and Bang, 1964a, 1968; Solum, 1970; Young, Levin and Prendergast, 1971), but the relationship of endotoxin clottable material to the hemostatic mechanism of *Limulus* has not yet been elucidated. Past studies of amebocyte aggregation employed methods that are essentially non-quantitative and utilized reagents that shed little light on the mechanism involved (Copley, 1947; Morrison and Rothman, 1957). With the exception of the possible participation of sulfhydryl groups (Bryan, Robinson, Gilbert and Langdell, 1964) little information is available on the components involved in the mechanism of amebocyte aggregation.

In the current work an attempt is made to study the mechanism of amebocyte aggregation utilizing the turbidimetric method originally developed by Born (1962) and O'Brien (1962) to follow mammalian blood platelet aggregation. This method, when adapted for the study of amebocyte aggregation, provides a quantitative system that permits reproducible measurements. The present study reports on the retardation of *Limulus* amebocyte aggregation by ethylene diaminetetraacetate (EDTA) and the restoration of aggregation to EDTA treated amebocytes by certain preparations from *Limulus* hemolymph. Some of the properties of an aggregation promoting material in *Limulus* hemolymph are also discussed.

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Materials and Methods

Animals

All animals used in these experiments were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, and measured 6–11 inches across the carapace. Horseshoe crabs were kept throughout the winter in an 800 gallon artificial sea water aquarium maintained at 58° F, and were fed shelled mussels, *Mytilus edulis*.

Glassware

Glassware and needles that contacted the hemolymph were silicon coated with Siliclad (Clay Adams).

Reagents

Physiological saline. A Tris-buffered saline solution was modified from Bryan, Robinson, Gilbert, and Langdell (1964) by mixing 0.51 M NaCl in a 9:1 ratio with 0.05 M Tris-HCl buffer at pH 7.8. The Tris-HCl buffer to saline ratio was established as the minimum concentration of Tris-HCl that would maintain pH 7.8 throughout amebocyte aggregation and retraction. The final concentration of NaCl in this mixture was 0.49 M, and whenever possible all solutions were made up in physiological saline. Imidazole-buffered saline, artificial sea water, and Tris-buffered *Homarus americanus* Ringer's solutions (Welsh and Smith, 1960) were also tested as diluents for aggregating amebocytes but Tris-buffered saline was found to be superior.

Buffered ethylene diaminetetraacetate (EDTA). Disodium EDTA was adjusted to pH 7.8 with 0.5 M NaOH and then diluted to a final concentration of 0.1 M with physiological saline. Buffered sodium citrate and oxalate solutions were prepared by a similar procedure.

Apparatus and assay for aggregation

A Chrono-log Platelet Aggregometer was adapted for the study of amebocyte aggregation by addition of an overhead rotary motor equipped with a polyethylene paddle, and a reverse thermocouple to regulate the temperature of the curvette. Aggregation was measured as changes in turbidity at 610 nm and recorded as per cent transmission by means of an attached recorder usually run at 1 inch per minute. Saline (2.0 ml) was added to the cuvette and equilibrated to 15° C while being stirred at 300–335 rpm. Material to be tested for reversal of aggregation inhibition was diluted in saline and treated the same way. The recorder was run for 1 minute to establish a baseline transmission before the addition of amebocytes.

Hemolymph was routinely withdrawn from the articular sinus of the legs with a siliconized, 1 inch, 18 gauge needle. To observe uninhibited aggregation, 4 ml of physiological saline at 15° C were used as diluent for 1 ml of hemolymph, whereas in inhibition studies, 4 ml of "inhibitor" were previously drawn into the syringe. After the hemolymph—saline or hemolymph—inhibitor was mixed by inversion of the syringe, 1 ml of the mixture was discarded and approximately

0.3 to 0.8 ml was added directly to the cuvette. Less than 10 seconds elapsed between puncture of the articular sinus and the addition of amebocytes to the cuvette.

Because the concentration of amebocytes was not constant in samples of a given volume, a range of 0.3 to 0.8 ml of hemolymph was added from the syringe to the cuvette. This was done to make the final concentrations of amebocytes in the cuvette as nearly equal as possible. However, the final concentrations of inhibitor could only be calculated approximately. For example, 10^{-1} M EDTA was diluted to 80 mM with hemolymph, but after the additional dilution in the cuvette, the final concentration of EDTA was approximately 17 ± 6 mM. Similarly, the agents tested for reversal of aggregation inhibition were diluted again by 0.3 to 0.8 ml of hemolymph in inhibitor. Unless stated otherwise, the range of concentrations indicates final, but approximate, concentrations.

Proportionality between O.D. at 610 nm and the concentration of unaggregated cells forms the basis of the turbidimetric method of measuring cell aggregation (Born and Cross, 1963). A standard curve relating the concentration of unaggregated amebocytes to O.D. 610 nm was prepared by counting the number of amebocytes treated with 10^{-1} M N-ethylmaleimide (NEM) at specific O.D.'s. In a given experiment, ameboycte aggregation in saline at 15° C was considered a control, the maximum aggregation for that particular animal. Quantitative comparison of aggregation data from different experimental samples or different animals is achieved by either of two methods: (1) Aggregation is expressed directly as changes in per cent transmission when the number of amebocytes in the samples is approximately equivalent. (2) In samples with different amebocyte concentrations, aggregation, recorded as per cent transmission, is converted to O.D. at 15 sec intervals and then expressed as a percentage of the control:

$$\frac{\Delta \text{ O.D.}_{\text{at time t/100 ambeocytes (experimental)}}}{\Delta \text{ O.D.}_{\text{ at time t/100 amebocytes (control)}}} \times 100 = \Delta\ \%_{\text{O.D.}}$$

Preparations

Serum. Serum was prepared from hemolymph (10 ml) withdrawn by cardiac puncture the prosoma-opisthosma junction with an 18 gauge, 1″ needle. The hemolymph was transferred from the syringe to 15 ml polytheylene tubes at room temperature. When retraction of the amebocyte aggregate was fairly complete (15 min), the cellular mass was removed and the resulting serum centrifuged at $1760 \times g$ for 5 min at 4° C to remove any residual cellular material. Serum is stable for 1 week or more at 4° C, and active for periods of 6 months or longer when stored frozen.

Cell free plasma. Cell free plasma was prepared by a procedure modified from Levin and Bang (1964a). Hemolymph (10 ml) was collected into an ice-cold syringe by cardiac puncture from a *Limulus* that was precooled at 4° C for 24 hours. Amebocytes were removed by centrifugation at 0° C in ice-cold centrifuge tubes until the centrifugal force equalled $12{,}100 \times g$. Approximately 8 ml of the resulting plasma supernatant was drawn off and stored either at 4° C or frozen.

Amebocyte homogenate supernatant (AHS). The amebocyte pellet from the preparation of cell free plasma was first washed twice, without resuspension, with

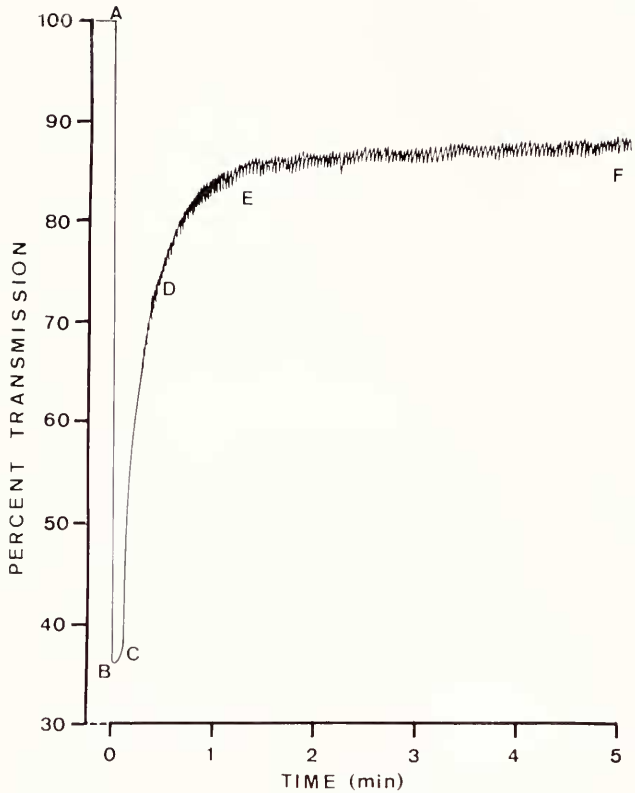


FIGURE 1. Typical photometric record of amebocyte aggregation at standard conditions. Physiological saline at 15° C is the diluent and stirring speed is 300–335 rpm. Records of aggregation show reproducible characteristics; decrease in transmission resulting from addition of amebocytes to the cuvette (A–B); a lag period (B–C); rapid rise in transmission (C–D); levelling off period (D–E); a plateau period (E–F). When transmission is converted to O.D. and aggregation is expressed as per cent change in O.D., the resulting curve has the same shape as that shown above.

1.0 ml cold saline and then frozen in 1.0 ml saline. After thawing, the pellet was homogenized by rotary motor at 1000 rpm or by hand for 5 min employing a ground glass homogenizer and teflon pestle. Freeze-thawing alternated with homogenization was repeated twice more. During homogenization, the homogenate was kept on ice and precautions taken to prevent foaming. The final volume of the homogenate was 2 ml, which represents approximately a 5-fold concentration of amebocytes in the original volume of hemolymph. The amebocyte homogenate was then centrifuged at 0° C at 12,100 × g for ten minutes. After centrifugation, the slightly opaque supernatant was drawn off and examined under a phase microscope to make certain that cellular fragments were absent.

The AHS yield was 1.5–1.8 ml for each 2 ml of homogenate and 0.5 ml aliquots were stored at –20° C.

Results

Characteristics of amebocyte aggregation in saline

When measured photometrically, amebocyte aggregation exhibits the characteristics shown in the typical aggregation graph in Figure 1. The sharp decrease in transmission that results from addition of amebocytes to the cuvette is followed by a short lag period, lasting fewer than 15 seconds, in which only a 0–2% rise in transmission occurs. Transmission rapidly rises following this lag period and 80–100 per cent of the total change in transmission takes place in the first 30 seconds. Over the next 30 sec, increases in transmission level off, and during the last 4 min, a plateau occurs where only slight changes in transmission are observed. Amebocyte aggregation can therefore be considered fairly complete at 1 min after

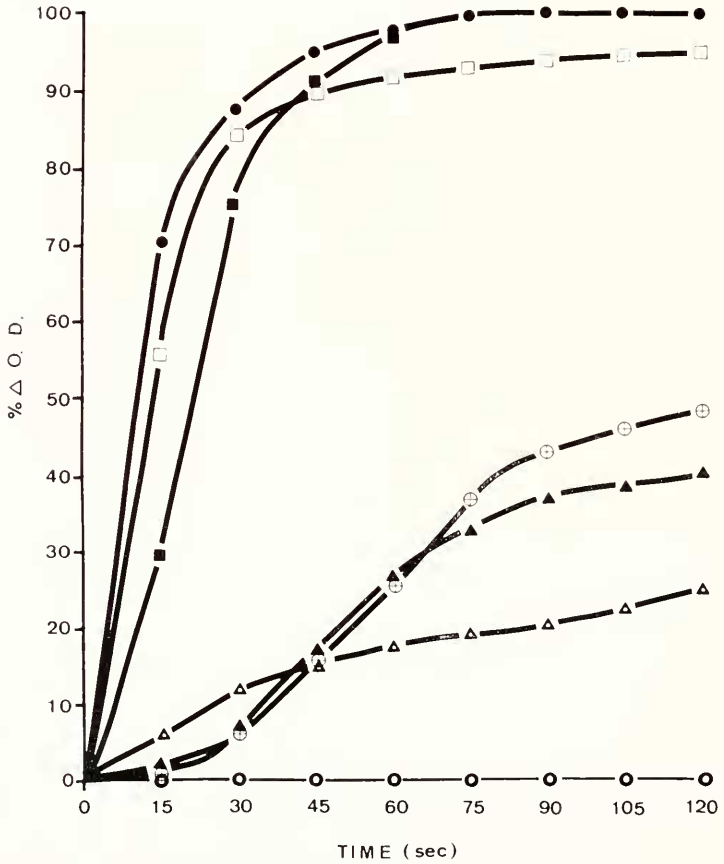


Figure 2. Typical inhibition of amebocyte aggregation by EDTA and citrate. Control aggregation in saline (●); amebocytes in: EDTA, 17 ± 6 mM (○); citrate, 17 ± 6 mM (△); EDTA, 8 ± 3 mM (▲); citrate, 8 ± 3 mM (⊕); EDTA, 2 ± 1 mM (□); citrate, 2 ± 1 mM (■). Concentrations of EDTA and citrate are approximate, but final concentrations in the cuvette.

the addition of amebocytes to the cuvette. During the plateau period, the recorder pen often oscillates with excursions of varying amplitude, presumably produced by free aggregates passing through the light path.

It was determined that the reproducibility of aggregation measurements is critically dependent on the temperature and the speed of stirring. Optimal aggregation occurs at 15° C and this temperature is routinely employed for measurement of amebocyte aggregation. Lower temperatures (10° C, 5° C, and 0° C) produce progressive inhibition of both the rate and extent of aggregation measured over 2 minutes. Microscopic examination of amebocytes at 0° C shows the presence of aggregates ranging in size from approximately 2–50 cells. Amebocytes within these aggregates retain their individual identity and pseudopods are only occasionally seen at the periphery of the aggregates. Stirring speed has a biphasic effect on aggregation. Low speed stirring (60–300 rpm) enhances the rate of

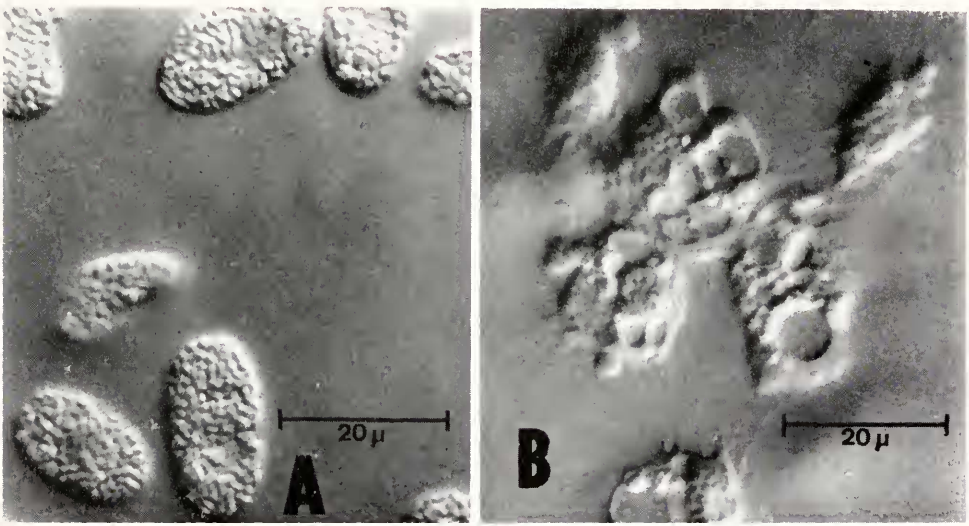


FIGURE 3. Photomicrographs of amebocytes 2 minutes after withdrawal into a, EDTA; b, saline (Nomarski Optics).

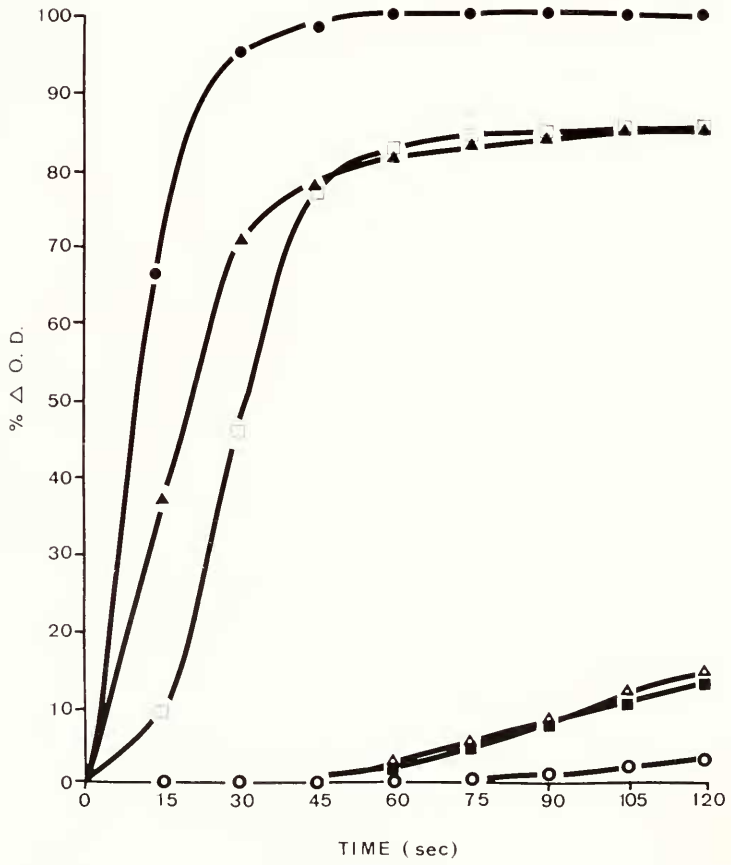
aggregation while higher stirring speeds (335–450 rpm) appear to inhibit aggregation. A stirring speed of 300–335 rpm is routinely employed. Variation in the number of amebocytes within that range suitable for photometric studies (700–1600 cells/mm^3) has no effect on the pattern of aggregation.

Inhibition of amebocyte aggregation by EDTA

Buffered EDTA at final concentrations of 17 ± 6 mM completely inhibits increases in transmission over the first two minutes (Fig. 2). In 8 ± 3 mM EDTA, inhibition of aggregation is significantly decreased and when the concentration of EDTA is reduced to 2 ± 1 mM, inhibition is negligible. Buffered sodium citrate at the same concentrations is less effective than EDTA, but inhibition of aggregation produced by both citrate and EDTA is concentration dependent (Fig. 2).

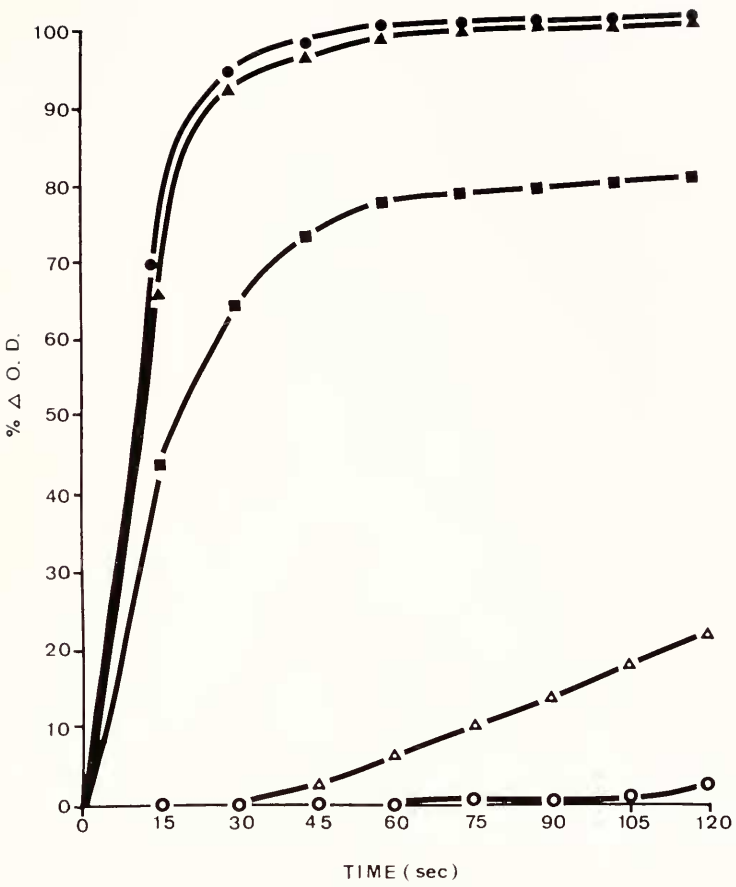
Oxalate inhibition of amebocyte aggregation is not suited to photometric examination, for, even at low concentrations (2 ± 1 mM), buffered oxalate causes expulsion of intact, free granules that produce turbidity and mask increases in transmission resulting from aggregation. As evaluated by microscopic observation, oxalate inhibition of amebocyte aggregation is intermediate between the inhibition produced by EDTA and citrate.

When examined microscopically amebocytes in 17 ± 6 mM EDTA appear intact and show little evidence of the extensive alterations characteristic of aggregation (Fig. 3). EDTA treated amebocytes remain ovoid and contain the characteristic granules (Fig. 3A), whereas, amebocytes in saline at 2 min (Fig. 3B)



4a.

FIGURE 4. Representative experiments showing the effect of calcium (4a) and magnesium (4b) on amebocytes in EDTA; (4a) control aggregation in saline (●), amebocytes in 17 + 6 mM EDTA (○); amebocytes in 17 + 6 mM EDTA added to: 8 ± 1 mM calcium (△); 15 ± 2 mM calcium (■), 24 ± 2 mM calcium (▲), 32 ± 3 mM calcium (□). (4b) Control aggregation in saline (●); amebocytes in 17 ± 6 mM EDTA (○). Amebocytes in 17 ± 6 mM EDTA added to: 8 ± 1 mM magnesium (△), 15 ± 2 mM magnesium (■), 24 ± 2 mM magnesium (▲).



4b.

FIGURE 4 (*continued*).

exemplify the typical morphological alterations of aggregation (Dumont, Anderson and Winner, 1966). Not infrequently, EDTA preparations have amebocytes that are more round than elliptical and a few free intact granules are present outside of the cells.

Although EDTA at 17 ± 6 mM markedly inhibits amebocytes aggregation, this inhibition is perhaps more aptly termed a retardation of aggregation because, with time, some aggregation takes place in EDTA. During the first 2 min after addition of amebocytes in EDTA to the cuvette, a slight rise (0–2%) in transmission frequently occurs, but over 5 min period increases in transmission are usually significant (0–15%). Furthermore, after stirring on the aggregometer for 5 min, microscopic examination of amebocytes in EDTA reveals aggregates even when no rise in transmission is observed. These aggregates range from small (approximately 2–20 cells) to medium sized (100 cells), and the amebocytes within these aggregates retain their identity with pseudopods evident only on those cells at the periphery of the aggregates. Lower concentrations on EDTA (in the range

of 14 ± 5 mM) are often as effective as 17 ± 6 mM EDTA in inhibiting amebocyte aggregation for 5 minutes but EDTA at final concentrations of 17 ± 6 mM is employed routinely.

The transistory nature of EDTA inhibition eliminates the possibility of employing standardized cell pools of known amebocyte number in aggregation experiments, for even if EDTA preparations are kept on ice with very gentle agitation to prevent sedimentation, most of the amebocytes are in small to medium sized aggregates within 15–20 minutes.

Effect of calcium and magnesium of amebocytes in EDTA

Calcium and magnesium produce aggregation of amebocytes in EDTA (Fig. 4). Minimum final concentrations of 8 ± 1 mM calcium or magnesium cause only slight

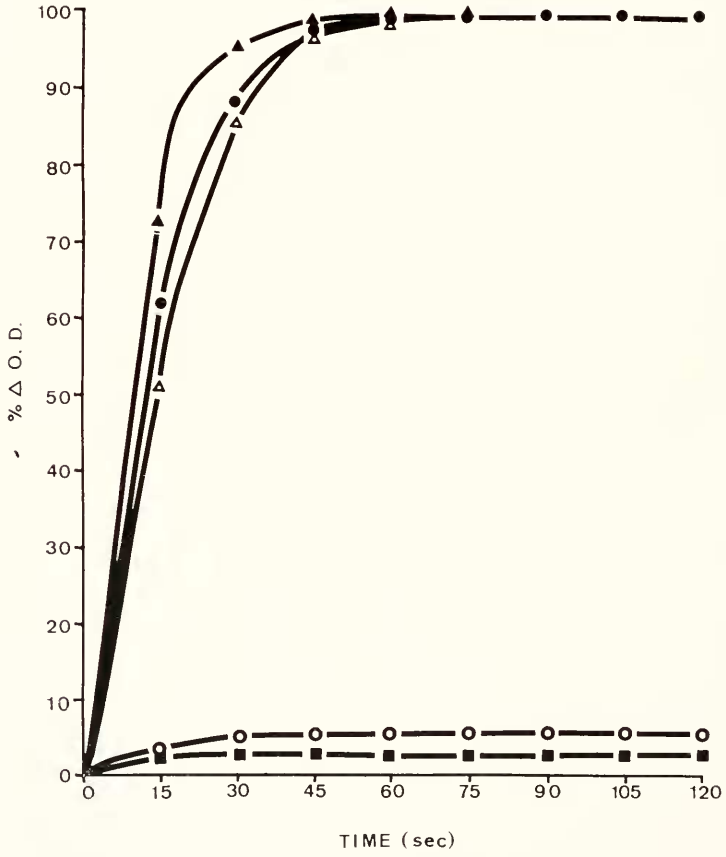


FIGURE 5. Effect of serum, AHS and cell free plasma on amebocytes in EDTA. Data from experiments on 3 different animals. Control aggregation in saline (●). Amebocytes in 17 ± 6 mM EDTA (○); amebocytes in EDTA with 1:20 diluted AHS (△); amebocytes in EDTA wtih 1:20 diluted serum (▲); amebocytes in EDTA with 1:20 diluted cell free plasma (■).

aggregation of amebocytes in EDTA but when magnesium levels are raised to 24 ± 2 mM, aggregation is complete within 30 sec. At similar concentrations, magnesium is more effective than calcium in reversing EDTA inhibition; calcium at 32 ± 3 mM produces only partial aggregation of amebocytes in EDTA. Potassium chloride in final concentrations of up to 32 ± 3 mM does not cause aggregation of EDTA inhibited amebocytes. Based on the results of 7 experiments that are similar to those shown in Figure 4, at least equivalent, if not excess magnesium is apparently required to reverse EDTA inhibition of amebocyte aggregation.

Reversal of EDTA inhibition

Addition of amebocytes in EDTA to a 1:20 dilution of either serum or Amebocyte Homogenate Supernatant (AHS) results in immediate aggregation of ameboyctes in EDTA (Fig. 5). The fall in optical density accompanying aggregation of amebocytes in EDTA by AHS or serum is indistinguishable in rate and extent from control aggregation in saline. Comparable dilutions of cell free plasma do not produce significant aggregation of EDTA inhibited cells.

Reversal of EDTA inhibited amebocyte aggregation by serum and AHS is not dependent either on the actual removal of EDTA, or on the effective removal of the chelating agent by addition of excess calcium or magnesium. The data in Figure 6 shows that if amebocytes withdrawn in EDTA are separated by gentle centrifugation at 4° for 30 sec at $280 \times g$, and then resuspended in fresh cell free plasma at 15° C, slow but spontaneous, aggregation takes place. When these resuspended, EDTA treated amebocytes in plasma are added to a 1:20 dilution of serum, aggregation is identical to control aggregation in saline. However, addition of amebocytes in EDTA to a 1:20 dilution of AHS or serum (not shown in Fig. 6) also produces aggregation that is indistinguishable from control aggregation.

The effect of temperature on serum induced aggregation of amebocytes in EDTA is similar to the effect of temperature on aggregation in saline; the temperature optimum for both is 15° C, and with lower temperature there is a concommitant decrease in the rate and extent of aggregation. At 0° C, serum—EDTA amebocyte samples consistently show a considerable lengthening of the lag period to 1 min or longer. Microscopic examination of these samples reveals small aggregates of amebocytes that retain their identity.

Some properties of serum and AHS

The potency of serum from different animals varies. Good reversal of EDTA inhibition is observed with dilutions of serum as great at 1:400 but, in general, the activity of serum begins to decline when dilution with saline is greater than 100-fold. AHS can often be diluted as much as 800 times before any decrease in aggregating activity is observed.

Samples of serum and AHS frozen for as long as 8 months still retain excellent activity. After 5 days at 4° C, activity of serum is generally unchanged, but AHS activity is diminished and a white insoluble precipitate forms within 2–12 hours at 4° C or after refreezing. Activity of serum and AHS is reduced or lost completely within 3–10 hours at room temperature. After 5 minutes in boiling water,

serum is completely inactive, but AHS at 1:10 dilution is observed to retain some very slight portion of activity. The aggregating activity of serum is unchanged after exposure to unsiliconized glass beads, and after prolonged dialysis at 4° C against physiological saline. Dialysis usually results in some reduction of the activity of AHS. Quantitative effects of dialysis on AHS are difficult to assess, however, because of the precipitate formed in the process of dialysis. The activity of serum or AHS is essentially non-dialyzable and heat labile. Therefore, the ability of these preparations to reverse EDTA inhibition of aggregation is not simply a result of the reversal of the effects of EDTA by free calcium and/or magnesium.

Aggregation promoting material: evidence for release and participation in amebocyte aggregation

Several experiments indicate the aggregation promoting activity of serum is produced by a substance (or substances) released from amebocytes during aggregation. If amebocyte aggregation is retarded by low temperatures, the supernatant (cell free plasma) does not cause aggregation of amebocytes in EDTA. Moreover, titration of the sera formed in aliquots of a hemolymph sample shows that as amebocytes aggregation proceeds with time in unagitated samples (up to 5 hours) there is increased aggregating activity in the serum.

Although exogenous cell free plasma has no effect on control aggregation in saline, the influence of plasma on the aggregating activity of AHS was examined. AHS is titrated to the lowest dilution that exhibits submaximal activity (1:800) and then incubated with varying concentrations of cell free plasma. No effect in the activity of the diluted AHS is noted after incubation with as much as 4 volumes cell free plasma.

The aggregation promoting material (APM) in serum and AHS may participate in amebocyte aggregation. When amebocytes in saline are added to a 1:20 diluted serum prepared from the hemolymph of another *Limulus*, aggregation is enhanced compared with control aggregation in saline alone. Withdrawal of hemolymph directly into dilute heterologous serum also enhances aggregation. The increase in aggregation by exogenous APM implies that endogenous APM may operate in the mechanism of amebocyte aggregation.

Mode of action of EDTA inhibition

To test for a direct effect of EDTA on the aggregation promoting material, amebocytes treated with EDTA are added to a 1:20 dilution of serum and increasing concentrations of EDTA (4 ± 1 mM to 71 ± 6 mM). Under these conditions, there is a progressive decrease in aggregation of EDTA cells that is correlated with increasing EDTA concentration. This decrease in restorative activity indicates that EDTA inhibits the aggregating activity of serum. But when serum, treated with an equal volume of 100 mM EDTA, is dialyzed against saline at 4° C, there is no effect on the aggregating activity of the serum. Therefore, if EDTA does directly inhibit the activity of the aggregation promoting material, such inhibition results in no permanent loss of activity.

To examine the possibility that EDTA could retard amebocyte aggregation

by preventing release of the aggregation promoting material from amebocytes, several approaches were employed. First, an attempt was made to isolate the active material from amebocytes treated with EDTA. Hemolymph was withdrawn into 100 mM EDTA so that the final concentration with amebocytes was 75 mM. After separation of the amebocytes by centrifugation, the EDTA—plasma was drawn off and the amebocyte pellet was washed and AHS prepared. The supernatant of this homogenate produces reversal of EDTA inhibition, and despite an extended lag period, the aggregation is complete. From these results it appears that amebocytes in EDTA do contain the APM.

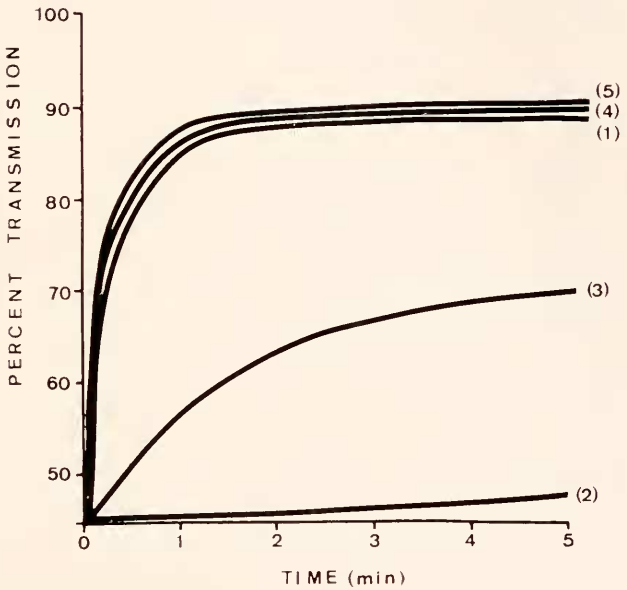


FIGURE 6. Tracings of representative graphs showing aggregation of EDTA inhibited amebocytes. Graphs are modified to smooth out oscillations; control aggregation in saline (1); amebocytes in 17 ± 6 mM EDTA (2); amebocytes withdrawn in EDTA and resuspended in cell free plasma (3); amebocytes withdrawn in EDTA, resuspended in cell free plasma, and added to 1:20 dilution of serum (4); amebocytes in EDTA added to 1:20 dilution of AHS (5).

A second approach employed to investigate possible inhibitory effects of EDTA on the release of APM from amebocytes involved direct assay of the supernatants of EDTA treated amebocytes. The ability of these supernatants to restore aggregation to amebocytes in EDTA is compared to that of the control supernatants of amebocytes treated with saline. Hemolymph is withdrawn in a 1:3 ratio with 100 mM EDTA, mixed and the suspension divided into equal aliquots in polyethylene centrifuge tubes. After the amebocytes are separated by centrifugation, the EDTA-hemocyanin supernatant is carefully removed, and the pellet of EDTA treated amebocytes covered with 0.2 ml cell free plasma and 0.8 ml physiological saline at room temperature. A total of 4 min elapsed between the time of puncture and the replacement of the supernatant by saline—plasma. The cell pellets are left in contact with the plasma—saline supernatants at room temperature for

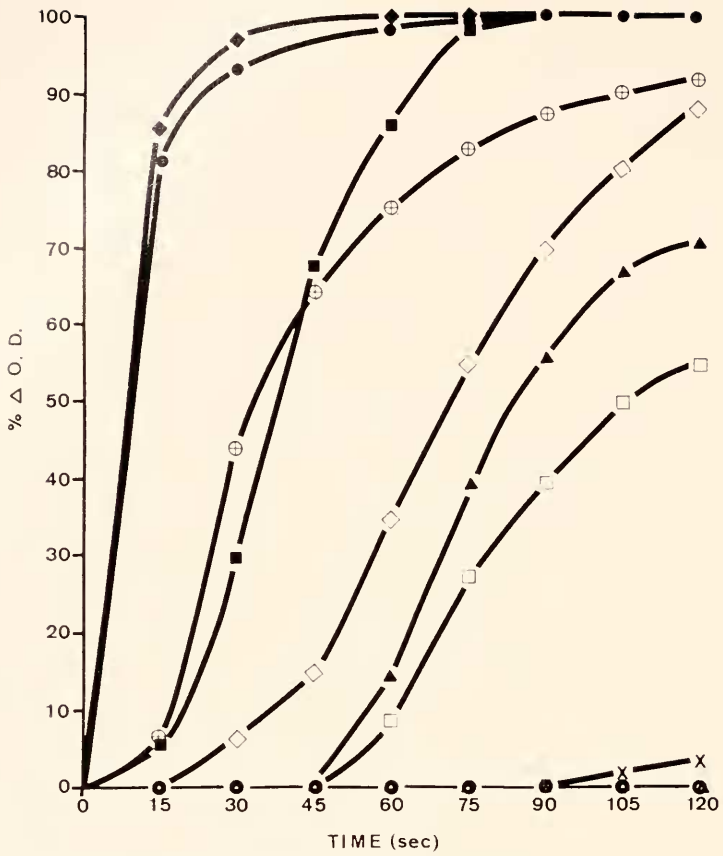


FIGURE 7. Typical experiment showing the effect of EDTA on the release of aggregation promoting material from amebocytes. Control amebocyte pellet represents amebocytes withdrawn in saline. EDTA amebocyte pellet represents amebocytes withdrawn in 75 mM final EDTA. At 0 time (4 min after puncture) supernatants of both pellets contained 0.2 ml inactive plasma + 0.8 ml saline, and did not produce significant aggregation of amebocytes in EDTA (×); aggregation in saline (control) (●); amebocytes in 17 ± 6 mM EDTA (○). Amebocytes in EDTA added to 1:20 diluted supernatants of: control amebocyte pellet at 1′ (▲); control amebocyte pellet at 10′ (■); control amebocyte pellet at 20′ (◆); EDTA amebocyte pellet at 1′ (△); EDTA amebocyte pellet at 10′ (□); EDTA amebocyte pellet at 20′ (◇); EDTA amebocyte pellet at 30′ (⊕).

periods of 1, 10, 20, and 30 min, at which times the supernatants are removed, checked microscopically to insure the absence of cells or cell fragments, and assayed for ability to restore aggregation to amebocytes in EDTA. At each time interval, the activity of the supernatants of EDTA treated pellets is compared with the control, saline—plasma supernatant of amebocytes originally withdrawn in a 1:3 ratio with saline. The results of a representative experiment (Fig. 7) show that at any given time the supernatant of the control amebocyte pellet is significantly more active in producing aggregation of amebocytes in EDTA than the supernatants of the EDTA treated amebocyte pellet. Reversing activity is present in

the control supernatant at 1 min, and at 20 min, aggregation of amebocytes in EDTA induced by this supernatant is equivalent to control aggregation in saline. However, activity only becomes evident in the supernatant of the EDTA treated pellet at 10 min, and not until 30 min did this supernatant produce complete but slow aggregation of amebocytes in EDTA.

The results of this experiment suggest that exposure to EDTA retards the release of APM from amebocytes. It could be argued that residual EDTA in the amebocyte pellet causes direct inactivation of APM and produces this effect, but this experiment has been performed with the same results using once-washed, EDTA treated amebocytes. The pellet method is preferred for this type of experiment to minimize disruption of amebocytes caused by resuspension.

Discussion

Amebocyte aggregation followed photometrically shows the basic characteristics of platelet aggregation (Skoza, Zucker, Jerushalmy and Grant, 1967). As a measure of aggregation, increases in transmission are quantitative and highly reproducible, but in so far as the manner in which these changes in transmission depend on the size and number of platelet aggregates, the photometric method remains empirical. From a study of the relationship between aggregate size and number and the observed changes in transmission, Born and Hume (1967) concluded that the relationship between platelet aggregation and observed changes in transmission involved both the formation of aggregates and the increasing density (contraction) of the aggregates. In view of the similarity between photometrically measured platelet and amebocyte aggregation, contraction of large amebocyte aggregates may be responsible for the characteristic rapid rise in transmission. Combination of single amebocytes to form small aggregates and formation of larger aggregates from small amebocyte aggregates would take place in the lag phase where transmission changes are slight.

In agreement with previous reports (Loeb, 1927; Copley, 1947) it was demonstrated that low temperatures retard the aggregation of *Limulus* amebocytes. Microscopic examination of amebocytes in saline reveals, however, that some aggregation can still take place at 0° C. In light of the findings of Born and Hume (1967), it might be assumed that aggregate contraction that would produce rapid increases in transmission is more affected by low temperatures than is the cohesion of individual amebocytes.

As a system for the study of the mechanism of amebocyte aggregation, inhibition by EDTA is somewhat limited in that aggregation does eventually occur, but EDTA is superior to the only other known aggregation inhibitor, N-ethylmaleimide, as the latter produces irreversible inhibition of amebocyte aggregation (Byran, Robinson, Gilbert and Langdell, 1964; Levin and Bang, 1966). Nevertheless, EDTA treated amebocytes are morphologically intact, usually remaining as single cells for 2 min or longer when stirred, and aggregate in a manner indistinguishable from aggregation in saline when diluted preparations of serum or AHS are added.

It is likely that EDTA "halts" amebocyte aggregation in its early stages rather than prevents the initiation of aggregation. When amebocytes are withdrawn in saline and then added to EDTA (or citrate) at a final concentration of

79 ± 7 mM, some aggregation takes place in the first 15 seconds, but after this time aggregation is significantly retarded, (unreported observations). Indeed, Dumont, Anderson and Winner, (1966) clearly demonstrated that in samples of aggregating amebocytes all cells do not undergo aggregation simultaneously, and that as late as 30 minutes after withdrawal of the hemolymph there were a few amebocytes that still remained ovoid and possessed characteristic cystoplasmic granules.

It should be made clear that because there is no fibrous protein of plasma origin analogous to the vertebrate fibrinogen—fibrin conversion in the hemostatic mechanism of *Limulus* (Howell, 1885; Loeb, 1903, 1927), a serum by traditional definition, does not form (Maluf, 1939). However, since the amebocyte cellular mass ("pseudocoagulum") retracts, consolidating the coaglum and squeezing out the non-cellular hemolymph, the term serum is used to describe the hemocyanin containing fluid remaining after amebocytes have aggregated and retracted. The supernatant of aggregated, retracted amebocytes has also been termed "pre-gel" by Levin and Bang, (1964a), but this term appears to be limited.

An aggregation promoting material may be involved in the mechanism of amebocyte aggregation. Not only is the rate and extent of serum restored aggregation of EDTA treated amebocytes strikingly similar to aggregation in saline but the temperature optimum for both types of aggregation is 15° C. Moreover, lowered temperatures inhibit both serum-restored and control aggregation in saline. Morphological observations of amebocyte aggregation in either saline or EDTA-serum preparations at 0° C suggest that aggregate contraction is more effectively inhibited by low temperatures than is the cohesion of individual amebocytes to form aggregates. Enhancement of aggregation in saline by additional serum directly supports the involvement of endogenous aggregation promoting material in the mechanism of amebocyte aggregation. Furthermore, when the amebocytes of an occasional *Limulus* failed to aggregate in saline, addition of active serum prepared from the hemolymph of another *Limulus*, without exception, immediately restored aggregation to these deficient amebocytes.

Inhibition of amebocyte aggregation by EDTA (and citrate) implicates calcium and/or magnesium in the events leading to amebocyte aggregation. Divalent cations, particularly calcium, seem to be required in the mechanism of cell aggregation of some other invertebrate species which, like *Limulus*, rely on the aggregation of hemostatic cells to halt the flow of blood from injured tissues (Bookhout and Greenburg, 1940; Boolootian and Giese, 1959; Noble, 1970). Calcium is essential for mammalian blood platelet aggregation induced by ADP, and calcium binding compounds such as EDTA, citrate, oxalate, and EGTA (ethyleneglycol diaminoethyl tetraacetate) inhibit this reaction (Born and Cross, 1963; Hovig, 1964; Skoza, Zucker, Jerushalmy and Grant, 1967; Ardlie, Nishizawa and Guccione, 1970).

At equivalent concentrations, EDTA is more effective in retarding amebocyte aggregation than citrate but the retardation of aggregation produced by both citrate and EDTA is concentration dependent (Fig. 2). Substantial differences in the stability constants for calcium ($10^{-10.59}$ for EDTA compared to $10^{-3.22}$ for citrate; Des Prez, Bryant, Katz and Brittinghan, 1967) and the fact that EDTA chelates both calcium and magnesium could explain the increased effectiveness of EDTA in inhi-

biting aggregation. Upon addition to equvalent, if not excess magnesium, amebocytes in EDTA aggregate, whereas, only partial restortation of aggregation is produced by equimolar calcium (Fig. 4). Hovig (1964) observed a similar phenomenon in EDTA treated rabbit platelets and postulated that addition of magnesium to EDTA preparations could liberate substantial amounts of ionized calcium from Ca · EDTA complexes by virtue of the ionic equilibria involved.

However, if EDTA specifically and exclusively inhibits amebocyte aggregation by chelation of divalent cations, the restoration of aggregation to amebocytes in EDTA by aggregation promoting material in serum or AHS is difficult to explain. First, the aggregating activity of serum or AHS is essentially heat labile and nondialyzable. Furthermore, serum and AHS are fully active at dilutions 1 : 100 and 1 : 400, respectively. Such dilutions would lower calcium and magnesium in serum from the reported values of 10 and 46 mM (Robertson, 1970) to levels that are experimentally shown to be too low to cause any aggregation of amebocytes in EDTA (Fig. 4). Thus, restoration of aggregation to amebocytes in EDTA could not be produced solely by simple addition of divalent cations in serum and AHS. It appears then, that inhibition of amebocyte aggregation by EDTA may be the result of non-specific or generalized effects of EDTA on amebocytes, as well as the specific chelation of divalent cations (see Weiss, 1960).

Although information on non-specific effects of EDTA is sparse, it has been reported that EDTA can form metal chelates with trace, divalent ions and the rare earth metals (Johnson, 1955; according to Weiss, 1960). There is also evidence that EDTA produces effects on diverse biological systems that cannot be explained by simple chelation of ionized calcium, magnesium, or trace ions (Blithell, 1964; Levin and Bang, 1964a, Walters, 1969; Noble, 1970). Furthermore, Rossi (1967) demonstrated that EDTA treated human platelets exhibited increased consumption of glucose as well as an alteration in the synthesis of organic phosphates and suggested that a generalized effect of EDTA on some metabolic enzymes might account for this observation.

Whatever the mechanism of EDTA in producing retardation of amebocyte aggregation, it must be assumed that the aggregation promoting material in serum and AHS circumvents a physiological process, or processes, blocked by EDTA. Moreover, it appears that this process blocked by EDTA is probably an early event in aggregation rather than the initiation of aggregation. Three plausible explanations can be considered for the relationship between aggregation promoting material and EDTA in amebocyte aggregation:

(1.) EDTA could render amebocytes insensitive to aggregation promoting material present in preparations of amebocytes in EDTA. Addition of excess serum or AHS would then create an over-abundance of the aggregation promoting material plus small quantities of ions and competitively, or otherwise, override the EDTA inhibition. This explanation is, in part, contradicted by the observation that very dilute preparations of serum and AHS can cause rapid and complete aggregation of EDTA inhibited amebocytes.

(2.) EDTA could retard amebocyte aggregation by either preventing the formation of the aggregation promoting material, or directly inactivating the material itself. It appears that aggregation promoting material is released intact from amebocytes and the similar restorative properties of AHS and serum support this

evidence. Then, EDTA must act intracellularly to prevent the formation of aggregation promoting material. However, amebocytes treated with EDTA contain active, aggregation promoting material. It, therefore, does not seem likely that EDTA produces inhibition of amebocyte aggregation by preventing the formation of the aggregation promoting material. On the other hand, when amebocytes in EDTA are added to serum (diluted 1 : 20) plus increasing concentrations of EDTA, the aggregation progressively decreases with increases in the EDTA concentration. Yet, after dialysis, serum treated with EDTA is fully active. It is possible that EDTA might directly inactivate the aggregation promoting material, but such inactivation is readily reversible.

(3.) EDTA could retard the release of the aggregation promoting material from amebocytes. Additional aggregation promoting material in the form of serum or AHS would then cause aggregation by initiating or enhancing release of more aggregation promoting material by the EDTA inhibited amebocytes. This explanation is supported not only by the isolation of aggregation promoting material from amebocytes treated with EDTA but also by the direct evidence shown in Figure 7.

Due to the preliminary nature of the evidence reported here, no one of these three explanations can be completely eliminated, but the hypothesis that EDTA retards amebocyte aggregation by preventing the release of the aggregation promoting material from amebocytes is, at present, the most appealing for several reasons.

Release of intracellular constituents by mammalian platelets is acknowledged as a prerequisite for irreversible platelet aggregation (Mills and Roberts, 1967), and from extensive studies employing chelating agents, it is generally agreed that calcium participates in the thrombin induced release reaction of platelets (Grette, 1962; Hovig, 1964; Zucker and Jerushalmy, 1967). More specifically, Kinlough-Rathbone and Mustard (1971), have reported that intraplatelet calcium is essential for the release reaction, but external calcium is required for the maintenance of intracellular calcium levels. It has been shown that aembocytes also release intracellular constituents during aggregation (Loeb and Bodansky, 1926; Levin and Bang, 1964a and 1964b; Dumont, Anderson and Winner, 1966). Furthermore, calcium is implicated in the release of intracellular consituents by a variety of cell types (see review by Stormorken, 1969). In light of the above information and the evidence presented in Figure 7, it is quite possible that EDTA retards amebocyte aggregation by interfering with the release of aggregation promoting material from amebocytes. In support of the possible release inhibiting role of EDTA, it is perhaps significant that colchicine which inhibits the release reaction of platelets (White, 1969) enhances EDTA inhibition of amebocyte aggregation, and N-ethylmaleimide which inhibits both amebocyte aggregation (Bryan, Robinson, Gilbert and Langdell, 1964) and the release reaction of platelets (Harrison, Emmons, and Mitchell, 1966) clearly prevents the release of aggregation promoting material from amebocytes (unpublished observations).

The presence of aggregation promoting material within hemostatic cells is by no means a novel concept. The early observations of Tait and Gunn (1918) indicated that a coagulating factor was present within the explosive corpuscles of the crayfish. Upon contact with foreign surfaces, these explosive cells released coagu-

lating factor which in turn promoted plasma coagulation around the aggregating cells. In a review of studies on arthropod blood coagulation, Maluf (1939, p. 179) concluded that "clotting in most arthropods is probably evoked by a liberation of thrombin into the hemolymph as a consequence of the rupture of certain blood cells." Moreover, Born (1965) asserted that the transition between the adhesion of a few platelets at the specific site of injury and the formation of an effective platelet plug must involve some sort of chain amplification mechanism. Although a specific relationship has yet to be established, the release *in vivo* of such substances as ADP and vasoactive materials by aggregating platelets may be the mechanism which is responsible for thrombus formation (Mills, Robb and Roberts, 1968). Ultimate proof of the specific involvement of an aggregation promoting material in *Limulus* amebocyte aggregation awaits identification and characterization of this material. Furthermore, the specific site for EDTA inhibition of amebocyte aggregation remains a pressing question. Since there is no evidence which indicates that aggregation promoting material is consumed or inactivated during aggregation, a control mechanism must exist to limit aggregation to the site of injury. Most important, the mechanism which initiates the transformation of amebocytes from the circulating to the aggregating state remains completely elusive.

Summary

1. The photometric method for measuring cell aggregation was adapted to study the aggregation of the amebocytes of the horseshoe crab, *Limulus polyphemus*.

2. At 15° C and a constant strring speed, amebocyte aggregation showed reproducible characteristics. Lowering the temperature below 15° C decreased both the rate and extent of aggregation but some slight aggregation still occurred at 0° C.

3. Aggregation was markedly retarded by buffered EDTA. The retardation of aggregation was dependent on the concentration of EDTA. Equimolar magnesium restored full aggregation to amebocytes in EDTA, whereas, similar concentrations of calcium caused only partial aggregation of EDTA treated amebocytes.

4. Dilute quantities of the serum supernatant of aggregated amebocytes and the supernatant of homogenates of amebocytes isolated at 0° C caused immediate aggregation of ameboyctes in EDTA. No significant aggregation was noted when EDTA treated amebocytes were added to the cell free plasma of hemolymph withdrawn at 0° C.

5. Serum or amebocyte homogenate supernatants did not require additional calcium and/or magnesium to induce aggregation of amebocytes in EDTA.

6. The aggregating activity of serum and amebocyte homogenate supernatants was essentially non-dialyzable and heat labile.

7. The data indicate the presence of an aggregation promoting material within amebocytes that is released from the cells during aggregation.

8. The possible relationship between this aggregation promoting material and EDTA induced aggregation inhibition is discussed.

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